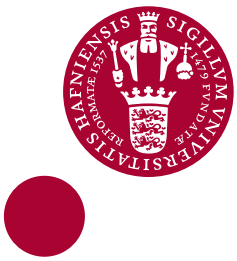

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Effect of ergosterol on the interlamellar spacing of deuterated yeast phospholipid multilayers

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ABSTRACT

Sterols regulate several physico-chemical properties of biological membranes that are considered to be linked to function. Ergosterol is the main sterol molecule found in the cell membranes of yeasts and other fungi. Like the cholesterol found in mammalian cells, ergosterol has been proposed to have an ordering and condensing effect on saturated phospholipid membranes. The effects of cholesterol have been investigated extensively and result in an increase in the membrane thickness and the lipid acyl chain order. Less information is available on the effects of ergosterol on phospholipid membranes.

Neutron Diffraction (ND) was used to characterize the effect of ergosterol on lipid multilayers prepared with deuterated natural phospholipids extracted from the yeast *Pichia pastoris*. The data show that the effect of ergosterol on membranes prepared from the natural phospholipid extract rich in unsaturated acyl chains, differs from what has been observed previously in membranes rich in saturated phospholipids. In contrast to cholesterol in synthetic phospholipid membranes, the presence of ergosterol up to 30 mol % in yeast phospholipid membranes only slightly altered the multilayer structure. In particular, only a small decrease in the multilayer d-spacing was observed as function of increasing ergosterol concentrations. This result highlights the need for further investigation to elucidate the effects of ergosterol in biological lipid mixtures.

1. Introduction

Sterols are fundamental cell membrane components, known for modulating its physico-chemical properties (Harayama and Riezman, 2018; Mouritsen et al., 2017). Sterols share a similar chemical structure as a result of the biological evolution of a common ancestor molecule, squalene (the acyclic sterol precursor), and its subsequent oxidation and cyclization in lanosterol (Bloch, 1983). The different sterols molecules can then be seen as the results of sequential chemical reactions (e. g. demethylation) leading to the transformation of lanosterol to the final sterol structure (Bui et al., 2016). In mammalian cells, cholesterol is the final product of this biosynthetic process and is present at up to ~ 50 mol% in the plasma membrane of higher vertebrates. For this reason, many studies have focused on understanding the role of cholesterol on the structure, dynamics and biological function of cell membranes (McMullen et al., 2004; Ohvo-Rekila et al., 2002; Rog et al., 2009).

Ergosterol is another biologically important sterol (Henriksen et al., 2006; Solanko et al., 2018); it is mainly present in fungi and protozoas and it is a direct or indirect target for many clinically available antifungal treatments (Ghannoum and Rice, 1999). Compared to cholesterol, ergosterol has an extra carbon-carbon double bond in the second sterol ring and a slightly different chemical structure of the acyl chain (Fig. S1). These structural differences were suggested to be responsible for the different effect of ergosterol and cholesterol on the physical properties of phospholipid membranes, such as membrane thickness and lipid acyl chain ordering (Hildenbrand and Bayerl, 2005; Shahedi et al., 2006).

Far fewer studies are available on the effect of ergosterol on the structure and dynamics of phospholipid bilayers compared to

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cholesterol. Furthermore, most of these studies have focused on the characterization of saturated phospholipid membranes, e.g. 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (Czub and Baginski, 2006; Pencer et al., 2005; Smondyrev and Berkowitz, 2001; Urbina et al., 1995) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (Bernsdorff and Winter, 2003; Cournia et al., 2007; Endress et al., 2002; Hsueh et al., 2005; Mannock et al., 2010; Tierney et al., 2005), by means of techniques such as Nuclear Magnetic Resonance (NMR), fluorescence spectroscopy, X-ray diffraction and Molecular Dynamics (MD). In saturated phospholipid membranes, ergosterol, as cholesterol, can induce a concentration dependent increase in the lipid acyl chain ordering and membrane thickness. In addition, the formation of a liquid-ordered phase, first identified in lipid membranes hosting cholesterol (Hjort Ipsen et al., 1987; Marsh, 2009), was also observed in membranes with an ergosterol concentration higher than 30 mol % (Bagnat et al., 2000; Mouritsen and Zuckermann, 2004; Shahedi et al., 2006). The overall ordering effect of ergosterol is found to be much smaller than that of cholesterol in 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (POPC) membranes, in contrast to the above-mentioned saturated phospholipid membranes (Henriksen et al., 2006; Hsueh et al., 2007). In this case, the concentration dependence of the ergosterol effect on the bilayer properties (e.g. bilayer thickness and rigidity) levels-off above 20 mol % of ergosterol, while a roughly linear dependence can still be observed for cholesterol at higher concentration (Arora et al., 2004; Henriksen et al., 2006). More recently, Hung et al. reported that when ergosterol is loaded into unsaturated lipid membranes, i.e.1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), its effect differs considerably from observations in DMPC membranes, and a bilayer thinning effect is observed, as opposed to thickening (Hung et al., 2016). Hence, while a universal model for sterol behavior in POPC bilayers was recently suggested (Henriksen et al., 2006), increasing the unsaturation level of the phospholipid acyl chain could lead to different effects.

Neutron and X-ray diffraction from stacks of lipid bilayers, i.e. lipid multilayers are capable of providing key structural information on lipid membranes (Buldt et al., 1979; Kucerka et al., 2008; Nagle and Tristram-Nagle, 2000; Zaccai et al., 1979). In these experiments, the diffraction of neutrons and X-rays from aligned lipid multilayers deposited on a solid substrate is used to determine the number and nature of the lipid phases present in the sample as well as structural information about the membrane thickness and internal structure (Foglia et al., 2015). The use of controlled humidity chambers allows monitoring the lipid multilayer structure at high relative humidity condition $\geq$ 98 % which mimics the physiological condition of biological membranes, as well as at lower relative humidity values. The investigation of the multilayer behavior as function of relative humidity and sample temperature can provide structural information on lipid phase transitions (Harper et al., 2001; Mills et al., 2009; Rappolt et al., 2003).

In neutron scattering experiments, as well as some NMR or IR-spectroscopy experiments, sample deuteration enables highlighting specific membrane components, and in neutron scattering experiments it can also improve the signal to noise ratio (Pabst et al., 2010). The extraction of deuterated lipids from microbial cell cultures, e.g. yeasts (de Ghellinck et al., 2014) or bacteria (Maric et al., 2015) grown in fully or partially deuterated media, is an efficient route for producing complex membrane biomimics composed of deuterated lipids. The use of such lipid membranes in different kinds of biophysical experiments, such as neutron scattering, however requires understanding of their physico-chemical properties (Bryant et al., 2019), which can differ from synthetic lipid and also influence the methods that can be used to prepare high-quality samples for structure determination.

Recently, the first characterization of lamellar membrane stacks prepared with the total lipid extract and phospholipid fraction from *Pichia pastoris* yeast cells grown either in deuterated or hydrogenous media was reported by our team (Gerelli et al., 2014; Luchini et al., 2018). Both the total lipid and the phospholipid extracts contain a range of different lipid species that reflect the natural lipid composition of *Pichia pastoris* cells. These studies showed that membrane stacks prepared with *Pichia pastoris* lipids are much more disordered than typical model membranes and highlighted the challenges involved in increasing membrane complexity towards more realistic cell membrane biomimics (Fragneto et al., 2018). As a continuation of these previous studies, a neutron diffraction investigation into the effect of ergosterol on *Pichia pastoris* phospholipid multilayers is presented here.

The total lipid extract of *Pichia Pastoris* is characterized by a low ergosterol content compared to the physiological amount found in the plasma membrane isolated from the same organism (Grillitsch et al., 2014). On the other hand, the fatty acid composition of the total lipid extract and the plasma membrane extract are overall very similar, although unsaturated fatty acids are slightly more abundant in the total lipid extract (Grillitsch et al., 2014; Klug et al., 2014). In this study, we separated the phospholipid fraction from the deuterated total lipid extract and added increasing amounts of either hydrogenous and deuterated ergosterol in a concentration range (i.e. 10 − 30 mol %), which is closer to the physiological ergosterol abundance in the *Pichia Pastoris* plasma membrane. The phospholipid fraction is lacking of relevant cell membrane components such as sphingolipids. Nevertheless, it still presents a higher level of complexity compared to commonly used biomembrane model systems, which typically include fewer phospholipid species, and at the same time it allows a direct comparison with previous investigations of ergosterol effect on saturated and unsaturated synthetic phospholipid membranes. The data show that loading ergosterol up to 30 mol % into the natural phospholipid multilayers has a very modest effect on the multilayer structure, and only a slight decrease in the multilayer repeat distance (i.e. d-spacing) was observed as the ergosterol concentration was increased.

2. Materials and methods

2.1. Chemicals

The deuterated lipid extract was obtained from *Pichia pastoris* yeast cells, GS115 (his4) purchased from Invitrogen grown in D_2O media as described below. Further purification allowed isolation of the deuterated natural phospholipids as well as deuterated ergosterol; hydrogenous ergosterol was purchased from Lordan. The lipid standards used for the analysis of the phospholipid extract were: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 2-Diacyl-*sn*-glycero-3-phospho-L-serine sodium salt from bovine brain (PS) and cardiolipin sodium salt from bovine heart (CL). POPE, POPS and CL and all the organic solvents were purchased from Sigma Aldrich (Sweden) and used without further purification. POPS and CL were dissolved in H_2O and extracted in chloroform before being used as TLC lipid standards. Analytical grade ($\geq$ 99 % purity) methyl pentadecanoate was purchased from Larodan Lipids (Sweden) and deuterated methyl pentadecanoate-*d32* was synthesized by the STFC Deuteration Facility at the ISIS Neutron and Muon Facility in Didcot (UK). H_2O was produced from a purifying system (MilliPore; resistivity >18 MΩ cm) and D_2O (> 99 % purity) was purchased from Sigma Aldrich (France).

2.2. Sample production

Pichia pastoris cultures were grown and the lipids were extracted as described in de Ghellinck et al. (de Ghellinck et al., 2014), and the lipid extract was dried under argon and stored at − 20 °C. The samples obtained correspond to the total lipid extracts, containing mainly phospholipids but also sterols, steryl-esters, free fatty acids and di-and triglycerides. In order to isolate the phospholipid fraction, the total extract was separated into the nonpolar and phospholipid fractions by flash column chromatography (silica, Roth, mesh size 0.04 - 0.063) using a chloroform/acetic acid solution, 100:1 v/v to elute the nonpolar lipids and methanol to recover the phospholipid fraction. Thin Layer

Chromatography with chloroform/methanol/water (65:25:4, v/v) as the solvent system, and I_2 vapor as the detecting agent, was used to check the quality of separation. A detailed description of the protocol used for the lipid analysis was previously reported (Luchini et al., 2018).

2.3. Lipid multilayer preparation

Thin silicon wafers (4 cm x 3 cm x 0.5 mm, <100> surface orientation) purchased from Silicon Materials (Germany), were cleaned by sequential sonication in chloroform, acetone, ethanol followed by plasma cleaning. We adapted a previously reported protocol (Himbert et al., 2017) to prepare the multilayers with the natural phospholipids. The deuterated phospholipid fraction (named hereafter dPol) and deuterated and hydrogenous ergosterol (named hereafter dErg and hErg respectively) were dissolved in a mixture of chloroform/methanol 2:1 (v/v) and subsequently dried in glass vials. The dried lipid films were resuspended and briefly sonicated in MilliQ water at a final concentration of 20 mg/ml. 900 µl of each solution were spread on the cleaned silicon wafers maintained horizontally. Drying proceed over 12 h under soft N_2 aeration. The wafers were subsequently placed under vacuum at 50 °C for at least 6 h followed by rehydration at 97 % RH for at least 24 h. Finally, the samples were placed in the humidity chambers, aligned, and equilibrated at 57 % relative humidity (RH). After measurements at this condition, the samples were equilibrated at 80 % and 98 % RH. The reservoir of the humidity chamber was filled with H_2O in order to guarantee the best contrast between the deuterated lipids and the hydration water.

2.4. Neutron diffraction experiment

Neutron diffraction data were collected at the cold neutron diffractometer of the Institut Laue-Langevin, D16 (Cristiglio et al., 2015), located in Grenoble, France. Neutrons with 4.5 Å wavelength were produced by reflection of the beam from a highly ordered pyrolytic graphite (HOPG) focusing monochromator. The sample to detector distance was 0.95 m and all samples were measured in reflection mode. The coated wafers were mounted vertically on a goniometer head placed in the top compartment of a humidity chamber (Gonthier et al., 2019). The temperature of the sample was maintained at 30 °C throughout the measurements while the temperature of the bottom compartment, corresponding to the reservoir of solvent, was adjusted according to the required relative humidity.

Diffraction data were collected at a detector angle (γ) of 12°, by scanning the sample angle (ω) in the range -1 to 10°, with a step of 0.05°. The neutron scattering intensity was recorded by a position sensitive two-dimensional ^{3}He detector (320 × 320) mm^2 area with a spatial resolution of 1 × 1 mm^2). Samples were first measured at 57 % RH and subsequently the RH was increased up to 98 % RH. Effective equilibration at 98 % RH was monitored by collecting ω-2θ scans until no changes were observed in the diffraction pattern. The equilibration at 98 % RH required at least 12 h.

2.5. Data reduction and analysis

Data reduction was carried out with the ILL software LAMP [Large Array Manipulation Program, http:/www.ill.fr/data_treat/lamp/lamp.html]. Background subtraction was carried out with a measurement of the empty humidity chamber scattering. The uniformity of the detector efficiency was calibrated with an H_2O scattering calibration file in LAMP.

The reduced 2D images were integrated in the ω range corresponding to the observed diffraction peaks in order to obtain intensity vs 2θ plots. The positions of the Bragg peaks in the plots were determined by fitting the peaks with a Gaussian function. The angular position of a Bragg peak is related to the scattering vector (q) value by Eq. 1:

$$q = \frac{4\pi}{\lambda}\sin\theta \quad (1)$$

The diffraction pattern produced by a lamellar phase composed of lipid bilayers alternating with water layers is characterized by Bragg peaks whose q-positions correspond to the characteristic ratio h/d, where h is the diffraction order and d is the lamellar spacing. Hence, from the q_1 and q_2 position of the first and second order Bragg peaks in the collected data, the characteristic lamellar d-spacing can be calculated according to Eq. 2:

$$d = \frac{2\pi}{q_2 - q_1} \quad (2)$$

The d-spacing corresponds to the unit cell thickness (one lipid bilayer and water layer). The errors reported for the d-values were obtained by propagation from the uncertainty in the q-positions estimated from the fit of the Bragg peaks.

Diffraction data collected for samples hydrated with different H_2O/D_2O mixtures as well as the pure D_2O and H_2O can be used to extrapolate the phases of the diffracted waves and hence to calculate the scattering length density distribution across the bilayers. The scattering length density distribution can provide information on how the molecules contained in the sample (in the present case phospholipids and ergosterol) are distributed in direction perpendicular to the substrate surface. However, this approach can only be applied when a sufficient number of diffraction orders (minimum 3) are observed. Because of the limited number of diffraction peaks produced by the natural lipid multilayers, ND data were collected in only one contrast (i.e. H_2O) and this work deals exclusively with the determination of the d-spacing. Although a molecular description of the natural lipid membrane cannot be directly extracted from the collected data, the d-spacing evaluation still allows a comparison between the overall structure, e.g. membrane thickness or number of lipid phases, of the multilayers prepared with different lipid composition. Further details on the contrast variation method as well as some applications can be found elsewhere (Foglia et al., 2015).

3. Results and discussions

Fig. 1 shows the diffraction data collected for the dPol multilayer without any ergosterol, which was used as a reference in this study. At 57 % RH the diffraction pattern contained 4 diffraction peaks, which were interpreted to belong to two different lipid phases. The d-spacing associated to each of these phases was calculated from the positions of the peaks associated to the first and the second diffraction orders and are summarised in Table 1.

Phospholipids can self-assemble into mesophases of different geometry, i.e. lamellar, hexagonal or cubic that can be identified by the relative 2θ positions of the Bragg peaks (Gunstone and Dijkstra, 2007; Tenchov et al., 2006; Williams et al., 1997). Lamellar phases are characterized by a series of equally spaced peaks corresponding to one dimensional periodicity. The first two diffraction peaks for the dPol multilayer, at 2θ 4.4 and 4.9 respectively, are too close to each other to correspond to the first and second diffraction order of any of the non-lamellar lipid phases (see Fig. S2), and therefore they originate in two different lamellar phases (named hereafter as phase a and b) in the direction perpendicular to the substrate surface.

Based on the lipid composition, the lamellar phase is also the most probable structural arrangement for the dPol sample. According to the dPol lipid composition analysis (Table S1-S2 and Fig. S3 - S4), the phospholipid mixture had a relatively low content of phosphoethanolamine lipids (PE ~15 %) and cardiolipin (DPG ~3.2 %), which could potentially form non-lamellar phases, especially at low relative humidity (Shyamsunder et al., 1988). In addition, the second order diffraction peaks for both phases are positioned at exactly twice the

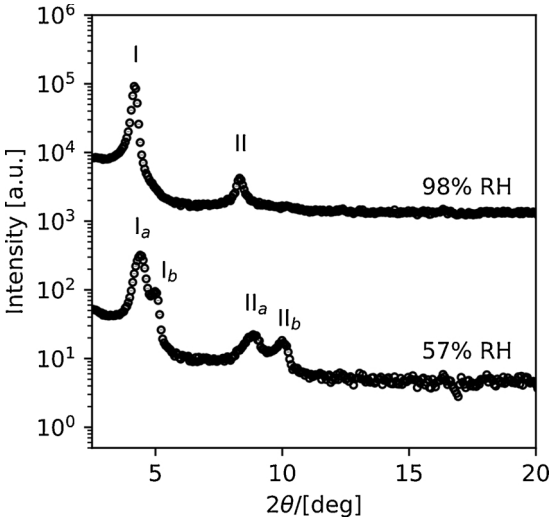

Fig. 1. Diffraction data collected at 57 % RH and 98 % RH for the dPol multilayer. Data collected at 98 % were scaled in order to allow a better comparison between the two data sets. The diffraction orders belonging to the same lipid phase are identified with roman numbers (i.e. I = first diffraction order, II = second diffraction order). The letters "a" and "b" are used to distinguish the diffraction peaks belonging to the two lipid phases detected in the sample at 57% RH.

Table 1
d-spacing values calculated from the Bragg peak positions in the collected diffraction data.

Sample composition	57% RH	98%RH
dPol	d_a = (58.5 ± 0.1) Å d_b = (51.4 ± 0.8) Å	d = (62.1 ± 0.6) Å
90 mol % dPol 10 mol % hErg	d_a = (55.6 ± 0.6) Å d_b = (51.4 ± 0.8) Å	d = (62.1 ± 0.8) Å
90 mol % dPol 10 mol % dErg	d_a = (57.2 ± 0.9) Å d_b = (55 ± 1) Å	d = (62.4 ± 0.2) Å
80 mol % dPol 20 mol % hErg	d_a = (61.5 ± 0.3) Å d_b = (50.7 ± 0.8) Å	d = (61.5= ± 0.7) Å
80 mol % dPol 20 mol % dErg	d_a = (60.1 ± 0.8) Å d_b = (49 ± 1) Å	d = (60.9= ± 0.7) Å
70 mol % dPol 30 mol % hErg	d_a = (57.9 ± 0.6) Å d_b = (48 ± 2) Å	d_a = (59.4 ± 0.4) Å d_b = (52.5 ± 0.5) Å
70 mol % dPol 30 mol % dErg	d_a = (55.8 ± 0.3) Å d_b = (50 ± 1) Å	d = (60.9= ± 0.3) Å

2θ − position of the first diffraction order, as expected for a lamellar phase. Nevertheless, the presence of only two diffraction orders does not allow to conclusively identify that both phases are lamellar (see also Materials and Methods section 2.5).

By increasing the relative humidity to 98 %, a structural rearrangement occurred and a single lipid phase was observed.The coexistence of different lipid phases was already reported in the case of lipids extracted from *Pichia Pastoris(*Luchini et al., 2018*)*. Because of the heterogeneous composition of these lipid mixtures (phospholipids with different headgroups and acyl chain length and unsaturation), we suggest that the phospholipid in the multilayer might separate in regions characterized by different d-spacing. In this previous study, a transition from a single lipid phase at 57% RH to two lipid phases at 98 % RH was reported (Luchini et al., 2018). Although the used dPol mixture had a similar lipid composition with respect to the dPol mixture used in the present study, the multilayers were prepared with different method. In the previous characterization (Luchini et al., 2018), the multilayer was prepared from a chloroform : isopropanol 1:4 (v/v) solution, while a water suspension of lipid vesicles was used to prepare the samples reported here (see Materials and Methods section). The lipid self-assembly in the lamellae forming the vesicles can potentially affect the multilayer structure. The preparation of lipid multilayers by vesicle deposition was previously reported (Del Favero et al., 2009) and differences in the multilayer structure were also observed for a DOPC multilayer prepared by drop casting of a either unilamellar or multilamellar vesicle suspension (Sironi et al., 2016). Although the calculated d-spacing values for the dPol multilayer (Table 1) are on average in agreement with the values previously reported (Luchini et al., 2018), the data clearly suggests that differences in the number of lipid phases and their behaviour with increasing RH might occur if an organic solvent solution or a water dispersion of lipids are used for the multilayer preparation. The same preparation method was adopted for all the samples reported here; hence the data can be directly compared to extrapolate the effect of the ergosterol molecules on the multilayer structure.

The presence of 10 mol % of either hErg or dErg in dPol did not dramatically affect the multilayer structure (Fig. 2). As for the dPol multilayer, two lipid phases where observed at low RH, which rearranged in a single phase at 98 % RH. An increase in the d-spacing was observed when the humidity was increased from 57 % RH to 98 % RH. However, the calculated d-spacing values did not show a significant difference compared to the dPol multilayer at either humidity levels. Very similar results were obtained for the multilayers prepared with dErg and hErg.

The diffraction pattern collected at 57% RH also showed an additional set of diffraction peaks. Sterols, and in particular cholesterol,

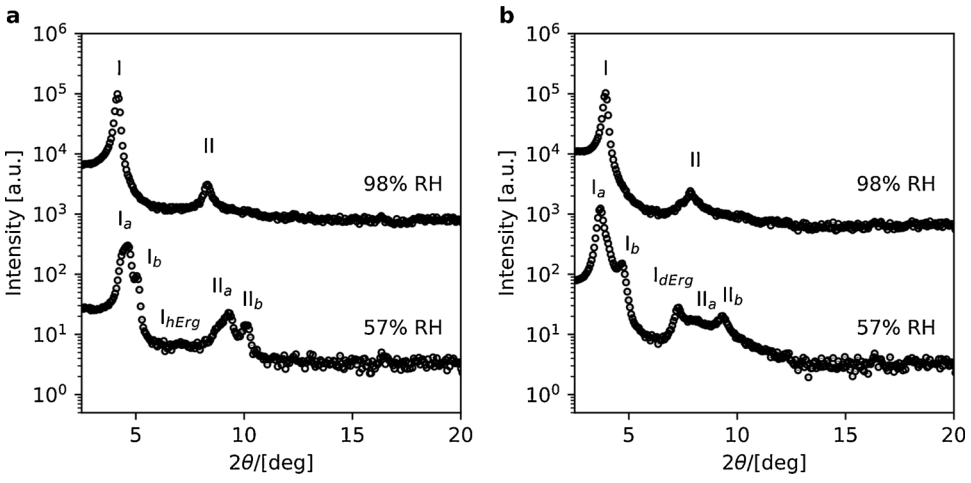

Fig. 2. Diffraction data collected at 57% RH and 98% RH for the 90 % mol dPol 10 % mol hErg (panel a) and 90 % mol dPol 10 % mol dErg 90:10 mol/mol (panel b) multilayers. In both graphs, data collected at 98% were scaled in order to allow a better comparison between the two data sets. The diffraction orders belonging to the same lipid phase are identified with roman numbers (i.e. I = first diffraction order, II = second diffraction order). The subscript "a" and "b" are used to distinguish the diffraction peaks belonging to the two lipid phases detected in the sample at 57% RH. "I_{hErg}" and "I_{dErg}" identify the first diffraction order of the ergosterol crystalline phase.

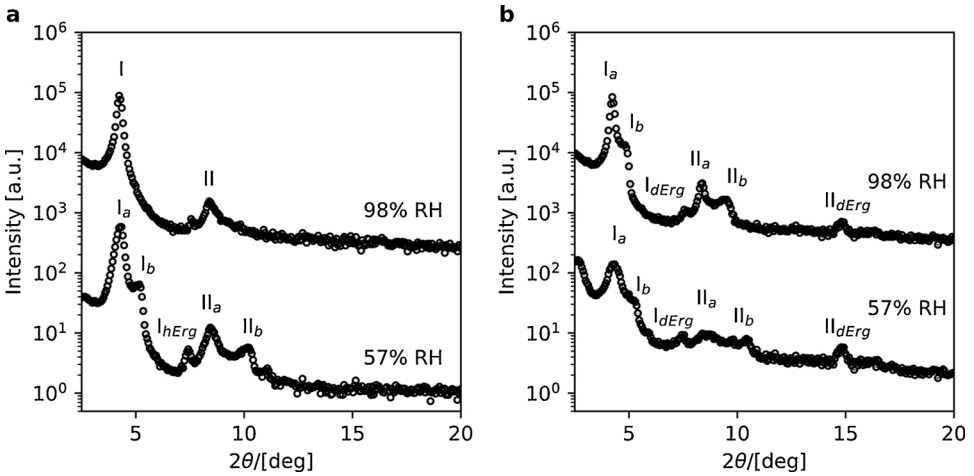

Fig. 3. Diffraction data collected at 57 % RH and 98 % RH for the 80 mol % dPol 20 mol % hErg (panel a) and 80 mol % dPol 20 mol % dErg (panel b) multilayers. In both graphs, data collected at 98 % were scaled in order to allow a better comparison between the two data sets. The diffraction orders belonging to the same lipid phase are identified with roman numbers (i.e. I = first diffraction order, II = second diffraction order). The subscript "a" and "b" are used to distinguish the diffraction peaks belonging to the two lipid phases detected in the sample. "I_{hErg}", "I_{dErg}" and "II_{hErg}", "II_{dErg}" identify the first and second diffraction order of the hydrogenous or deuterated ergosterol crystalline phase.

form a crystalline phase with a characteristic diffraction pattern (Hull and Woolfson, 1976; Loomis et al., 1979; Shieh et al., 1977). This crystalline phase has also been reported to coexist with the phospholipid lamellar phase when cholesterol molecules are not all included in the phospholipid bilayer, but are partially crystallized outside the bilayer (Bach and Wachtel, 2003). Similar results were also reported for ergosterol (Hung et al., 2016). The presence of the ergosterol crystalline phase was confirmed here by the 2θ positions of the additional set of diffraction peaks in the collected data. The rearrangement of the multilayer with increasing humidity might improve ergosterol solubilization in the bilayers and hence, the ergosterol crystalline phase was no longer detectable at 98 % RH.

Fig. 3 and 4 shows the data collected for the dPol multilayers with 20 mol % and 30 mol % ergosterol respectively. Two lipid phases could be observed at low RH also in this case, but for 20 mol % dErg and 30 mol % hErg the two phases were also present at 98 % RH. The intensity of the peaks belonging to different diffraction datasets is not always directly comparable. In fact, even if the same amount of lipids is deposited on the substrate, the intensity of the diffraction peaks is strongly dependent on the order in the sample, which in the case of natural lipids can be extremely variable and a parameter difficult to control. For this reason, the lower intensity of the peaks collected at 57 % RH in Fig. 4 compared to Fig. 3, which were assigned to the ergosterol crystalline phase, might not necessarily suggest a lower content of the ergosterol crystalline phase in the samples.

The results listed in Table 1 show that at 98 % RH the d-spacing calculated for dPol multilayer is (62.1 ± 0.6) Å, while the values for the dPol/hErg and dPol/dErg multilayers with 20 mol% ergosterol are (61.4 ± 0.3) Å and (60.9 ± 0.6) Å respectively. The reduction of the d-spacing at 98% RH was more evident for the multilayers with 30 mol % ergosterol: (59.4 ± 0.5) Å for dPol/hErg and (60.1 ± 0.3) Å for dPol/dErg.

Altogether, while different lipid phases were observed at low RH, a single predominant phase appeared to characterize most of the multilayers at 98%. The overall effect of dErg and hErg on the dPol multilayers at 98% RH is summarized in Fig. 5 where the d -spacing measured for the different samples is displayed as function of the ergosterol concentration. The results show that the loading of ergosterol up to 30 mol % in the dPol multilayer does not induce a large variation in the d-spacing, in contrast to what has been previously reported for cholesterol in different phospholipid systems (Hung et al., 2007). This result is in agreement with the previously reported characterization by neutron reflectometry of a deuterated bilayer prepared with phospholipids and ergosterol extracted from *P. pastoris*, although the ergosterol concentration was much lower (~5 % mol) (de Ghellinck et al., 2015b).

Fig. 5 shows a small d-spacing decrease as the concentration of both h and d ergosterol is increased. The observed change in the d-spacing might be associated with a variation in the lipid packing, which produces a decrease of the membrane thickness, or a variation of the thickness of the water layer between the bilayers; both of these effects might be induced by the presence of ergosterol molecules in the bilayers. Recently, Hung et al. investigated the effect of ergosterol on different synthetic phospholipid bilayers and observed a small decrease in DOPC membrane thickness, which was found to be dependent on the amount of the ergosterol in the bilayer (Hung et al., 2016). This effect was proposed to be related to the fact that ergosterol, unlike cholesterol, does not enable the phospholipid acyl chain tilt to decrease,

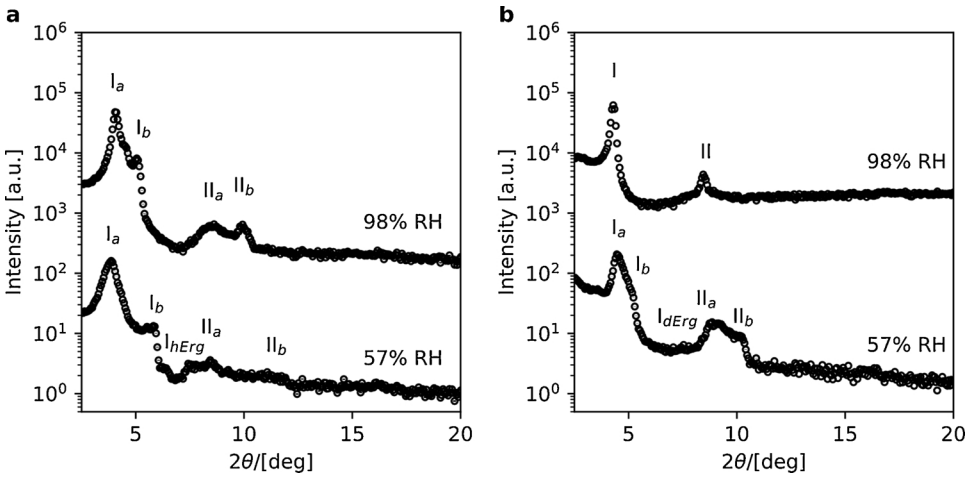

Fig. 4. Diffraction data collected at 57 % RH and 98 % RH for the 70 mol % dPol 30 mol % hErg (panel a) and 70 mol % dPol 30 mol % dErg (panel b) multilayers. In both graphs, data collected at 98 % were scaled in order to allow a better comparison between the two data sets. The diffraction orders belonging to the same lipid phase are identified with roman numbers (i.e. I = first diffraction order, II = second diffraction order). The subscript "a" and "b" are used to distinguish the diffraction peaks belonging to the two lipid phases detected in the sample. "I_{hErg}", "I_{dErg}" identify the first diffraction order of the hydrogenous or deuterated ergosterol crystalline phase.

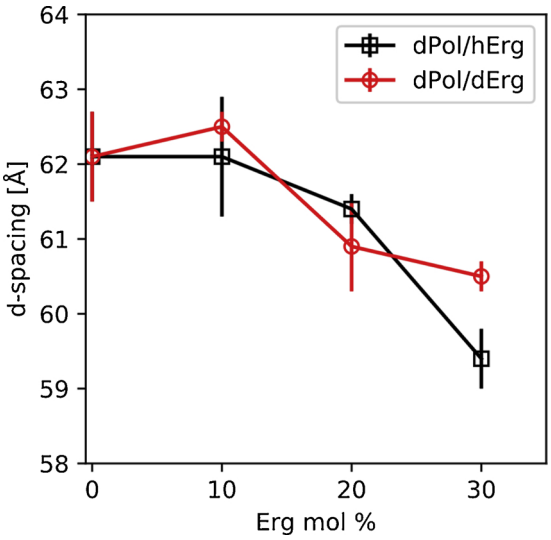

Fig. 5. d-spacing values calculated from the diffraction data collected at 98 % RH versus hydrogenous or deuterated ergosterol content in the multilayers.

which is reported to produce the membrane thickening in phospholipid multilayers prepared with cholesterol.

Overall, hErg and dErg induced similar d-spacing values for the lipid multilayer; however, a small difference was observed at 30 mol % of hErg and dErg. In this specific case, two lipid phases were still observed at 98 % RH only for hErg and a small difference in the d-spacing was calculated as 1.5 ± 0.7 Å for dErg and hErg (if phase a of dPol/hErg is taken into account). Compared to hydrogenous phospholipids, deuterated phospholipids have a lower chain melting temperature, which can have an effect on the bilayer physical properties at a given temperature (Bryant et al., 2019). Similar arguments could be used to explain the difference observed for the multilayers containing dErg and hErg, although a small effect of the sterol deuteration was only detectable when the sterol concentration was increased to 30 mol %.

4. Conclusions

Lipid multilayers were prepared by mixing deuterated phospholipid (dPol) extracted from *Pichia Pastoris* and ergosterol, with the aim to characterize the effect of the ergosterol concentration on the natural phospholipid membrane structure. A different sample preparation method was used in comparison to a previous study (Luchini et al., 2018), where the multilayers were formed *via* lipid deposition from a chloroform/isopropanol 1:4 (v/v) solutions. Nevertheless, the presence of many different phospholipid species (different acyl chain and headgroup composition) in the samples allowed only for a limited order in the lipid multilayers and hence only two diffraction orders were detectable. As a result of the complex composition of the dPol mixture, two lipid phases were observed at 57%RH. In agreement with our previous findings (Luchini et al., 2018), we hypothesize that different phospholipid species separates into regions of the multilayer characterized by different d-spacing. Although the multilayers did not exhibit a sufficiently ordered structure to allow the Fourier analysis of the diffraction patterns and hence a more detailed description of the membrane structure on the molecular level (see also Materials and Methods, section 2.5), changes in the characteristic membrane d-spacing could be used to observe changes in the overall multilayer structure.

The results suggest the presence of a crystalline ergosterol phase outside the membrane at 57 % RH (Hung et al., 2016). Interestingly, at 98 % RH the crystalline ergosterol phase was not detectable in most of the collected data. This observation is in agreement with previously reported neutron reflectivity data from samples in bulk water (de Ghellinck et al., 2015a; Fragneto et al., 2018), which indicated that up to 30 mol % ergosterol can be solubilized in a bilayer.

The effect of ergosterol on the lipid membrane structure was previously mainly investigated in phosphatidylcholine lipids with different unsaturation degree in the acyl chains (Florek et al., 2018; Pencer et al., 2005; Sabatini et al., 2008). Compared to these reports on synthetic lipid multilayers, dPol have a much more complex lipid composition, i.e. several different phospholipid headgroups and acyl chains. Nevertheless, the lipid composition analysis (see supplementary materials) showed that phosphatidylcholine headgroups (≥ 50 %) and unsaturated acyl chains (≥ 70 %) are most abundant in this sample. In this respect, it is interesting to note that our results suggest an effect of ergosterol on dPol multilayers very similar to the one recently observed by Hung et al. for DOPC lipid multilayers (Hung et al., 2016). Up to 30 mol % ergosterol has only a small effect on the multilayer structure and a small d-spacing reduction was observed only when the ergosterol content was increased to 30 mol %, 1.5 ± 0.7 Å for dErg and hErg.

Both fully deuterated and hydrogenous ergosterol were used. Similar results were obtained for both, although a slightly different d-spacing was observed at 30 mol % ergosterol concentration, which could in principle be related to the isotopic substitution in the sterol molecules.

Overall, the ergosterol did not strongly affect the dPol multilayer structure, in contrast to previously reported results for cholesterol in phospholipid multilayers (de Meyer and Smit, 2009; Hung et al., 2007). Nevertheless, a small decrease in the membrane d-spacing was detected, which was found to be dependent on the ergosterol content. This result highlights the need for further investigation of the structural and functional effects of ergosterol in complex biological membrane mimics as it considerably differs from what it is known for cholesterol in synthetic model membranes.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chemphyslip.2020.104873.

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